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THE EFFECTS OF TISSUE EXTRACTS AND EXPERIMENTAL GRANULOMATA ON RAT SERUM GLYCOPROTEINS

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THERE is now abundant evidence that the serum α_1-globulins, including those which are elevated in cancer and infection, are produced by the liver (Hochwald, Thorbecke and Asofsky, 1961 ; Sarcione, 1963 ; Miller, Hanavan, Titthasiri and Chowdhury, 1964). Burston, Tombs, Apsey and Maclagan (1963) suggested that in cancer the tumour influences the liver to produce more α_1-globulins by means of a humoral mechanism. It is still not known, however, whether the factor responsible for the increased synthesis of α_1-globulin arises from tissue growth (Burston, Apsey and Maclagan, 1965) or tissue destruction (Darcy, 1964), and often in the neoplastic and inflammatory conditions these coexist, making a clear distinction impossible.

The experiments reported here were an attempt to show that a humoral mechanism did exist, and that a factor could be extracted from Walker 256 carcinoma which, when injected into the rat, would cause an increase in the serum glycoprotein level. Although the evidence obtained for a humoral mechanism was equivocal, some light was thrown on the effect of tissue growth on the serum glycoprotein level in the absence of visible necrosis.

METHODS AND MATERIALS

Albino, male Wistar rats (average weight 180 g.) were obtained from A. Tuck and Son Ltd., Rayleigh, Essex. The animals were maintained on Oxo diet 41B and tap water *ad libitum*.

Preparation of tissue extracts.—Normal rat livers and Walker tumours were dissected out, pooled and homogenised for 1·5 min. in an Atomix blender (Measuring and Scientific Equipment Ltd., London) in a volume of cold 0·9 per cent (w/v) saline or cold distilled water equal to the wet weight of the tissue. Unless otherwise stated all extracts were centrifuged at 5,000 × ***g*** for 10 min. and then at 57,000 × ***g*** for 60 min. in an H.S. 40 centrifuge (Measuring and Scientific Equipment Ltd., London). Aqueous extracts were lyophilised and stored at 4°, saline extracts were stored at −20°.

Injection of tissue extracts.—Saline extracts were used directly, whilst lyophilised extracts (2 g.) were dissolved in 10–12 ml. of sterile saline. All solutions, including saline and rat

serum controls, were centrifuged at 105,000 × *g* to remove any undissolved material and bacteria. The extracts (in some cases mixed with adjuvants as described below) were then injected subcutaneously into the right flank of the rat using sterile disposable syringes and needles.

Freund's adjuvant.—To 4 ml. saline, serum or tissue extract, 2 ml. of Freund's incomplete adjuvant (Difco Ltd., Detroit, U.S.A.) was added and the mixture emulsified in an ultrasonic disintegrator (Measuring and Scientific Equipment Ltd., London) (1·5 amp. for 1 min.). Volumes (1 ml.) of the emulsion were injected into groups of 4 rats.

Aluminium hydroxide.—To 4 ml. saline, serum or tissue extract 360 mg. of Al $NH_4(SO_4)_2$ $12H_2O$ was added with warming, followed by 0·008 M NaOH (4 ml.) to give a precipitate of aluminium hydroxide. The coarse precipitate was resuspended by shaking and 1 ml. volumes were injected into groups of 4 rats.

Polyvinylpyrrolidone (PVP).—To 5·5 ml. of saline, serum and tissue extract 1·1 g. of PVP was added to give a final concentration of 20 per cent. These solutions (1 ml.) were injected into groups of 4 rats.

Protamine sulphate.—To 3 ml. of saline, serum and tissue extract, 3 ml. of 1 per cent protamine sulphate in saline (British Drug Houses Ltd., Poole, Dorset) was added. The small precipitate which formed with the tissue extracts was resuspended and 1 ml. portions were injected into groups of 4 rats.

Fibrinogen.—To 8 ml. of saline, tumour extract and bovine serum albumin (Nutritional Biochemical Co., Cleveland, Ohio, U.S.A.), bovine fibrinogen (Armour Ltd., Eastbourne, Sussex) was added to give a final concentration of 40 mg./ml. The solutions were centrifuged at 105,000 × *g* for 10 min. and 1 ml. portions were injected into groups of 4 rats. The syringe was removed leaving the needle *in situ* and 0·1 ml. of thrombin (23 units) was injected from a second syringe.

Collection of blood.—The rats were killed under ether anaesthesia and bled from the heart.

Removal of granulomata.—Where possible granulomata were dissected out, any enclosed unabsorbed material was removed, and the capsule was weighed on a Torbal DWL 3 balance.

Protein estimation methods.—Total serum protein, total liver protein, serum glycoprotein were determined as described previously (Burston, Apsey and Maclagan, 1965) except that in the serum glycoprotein estimation the serum was only diluted 0·1 to 1 ml. prior to the addition of perchloric acid.

Carbohydrate estimation method.—The seromucoid fraction was isolated, as described above, from 0·5 ml. of serum and the hexose content was determined by the method described by Winzler (1955), using galactose and mannose (1 : 1) as a standard.

RESULTS

Effect of injected tumour extracts on rat serum glycoprotein levels

In preliminary injection experiments, small doses (0·5 ml. per day) of nuclei-free Walker tumour extract, administered subcutaneously over a period of 10 days to imitate the effect of the tumour, had no effect on the serum glycoprotein (SGP) level. However, 4 ml. of tumour extract produced a marked rise 48 hr. after injection (Fig. 1a). In this experiment the extracts were centrifuged at 5,000 × *g* before injection in an attempt to remove bacteria, but in later experiments a higher centrifugal force was found to be necessary. The administration of 4 ml. of fluid subcutaneously was felt to be unphysiological, although the SGP levels of saline injected controls were unaffected by this volume. It was decided to reduce the dose volume, but keep approximately the same protein concentration by freeze drying the aqueous extract, redissolving it in a small volume of saline and centrifuging at 105,000 × *g* for 10 min. In order to determine if the response to tumour extract was specific, rat liver, homogenised in physiological saline, and rat serum were used as controls.

It can be seen from Fig. 1b that 1 ml. volumes of both liver and tumour extract produced a rise in the SGP level whereas rat serum had no effect.

Although in these experiments a factor in rat liver and in Walker tumour tissue increased the level of SGP in the injected animals, the actual rise produced was small compared with the increase produced by growth of the tumour *in vivo* (800 mg./100 ml.). Since the latter takes place over a longer period of time, the effect of various adjuvants known to delay absorption was investigated.

Experiments with Freund's adjuvant

The results of injecting saline and Walker tumour extract in Freund's adjuvant are shown in Fig. 1c. It can be seen that there was a marked increase in the SGP level in the tumour injected rats. The effect was, however, non-specific as both a bovine serum albumin and a rat serum produced similar results (Fig. 1d and e.).

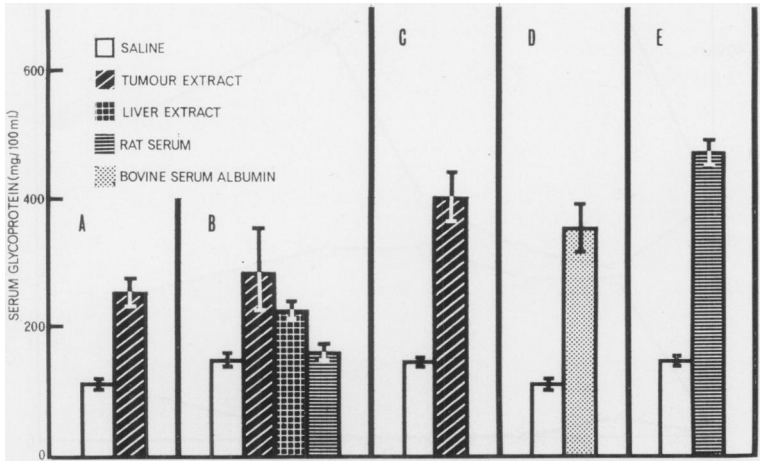


FIG. 1.—Serum glycoproteins 48 hr. after various subcutaneous injections. A. 4 ml. volumes, B. 1 ml. volumes without adjuvant. C, D and E. 1 ml. volumes in presence of Freund's adjuvant. Each column represents mean ± S.E. of groups of 4 animals.

On autopsy it was found that in the saline injected animals the emulsion had spread out to cover a large area of the subcutaneous tissue, whereas in the animals injected with tumour extract, rat serum, or bovine serum albumin, the material was completely encapsulated and was surrounded by the yellow jelly-like substance similar to that found surrounding the Walker tumour. It appeared therefore, that the encapsulation process was intimately involved with the rise in the serum glycoprotein level.

The time course of the two processes was investigated by injecting rat serum + Freund's adjuvant and saline + adjuvant mixtures followed by measurements at various intervals (Fig. 2). In the serum injected rats there was rapid growth of capsular material over the first 24 hr. but subsequently little further growth. The saline + adjuvant mixture produced little or no capsular material. The SGP curve for the serum injected animals was complex, there was a rapid increase over the first two days after injection followed by a fall on the 4th day and then a rise on the 6th day. The saline + adjuvant injected animals on the other hand showed only a small increase over the first 3 days after injection followed by a sharp rise to a maximum on the 4th day. This was not correlated with capsule

formation and it would appear that two mechanisms are in operation, the first a rapid increase in SGP in response to capsule growth and the second a slower effect involved in removing the adjuvant from the injection site.

Hexose content of seromucoid fraction

Macbeth and Bekesi (1964) indicated that the carbohydrate content of the seromucoid fraction which increased as a reponse to the Walker tumour was different from that produced by tumour injection. In order to confirm this,

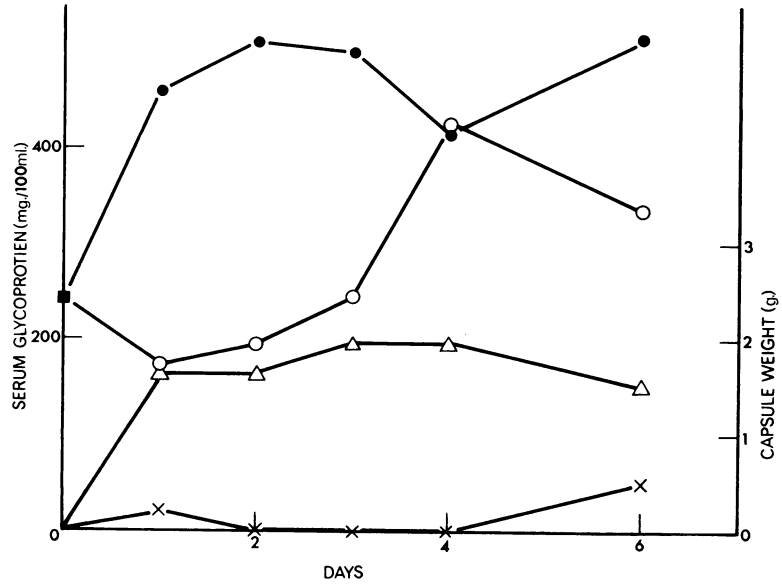


FIG. 2.—Serum glycoprotein response and capsule growth produced by subsutaneous injection of rat serum + Freund's adjuvant. Each point represents the mean of 3 animals.

●——● Rat serum + Freund's adjuvant
○——○ Saline + Freund's adjuvant
△——△ Capsule wt. (serum + adjuvant)
×——× Capsule wt. (saline + adjuvant)

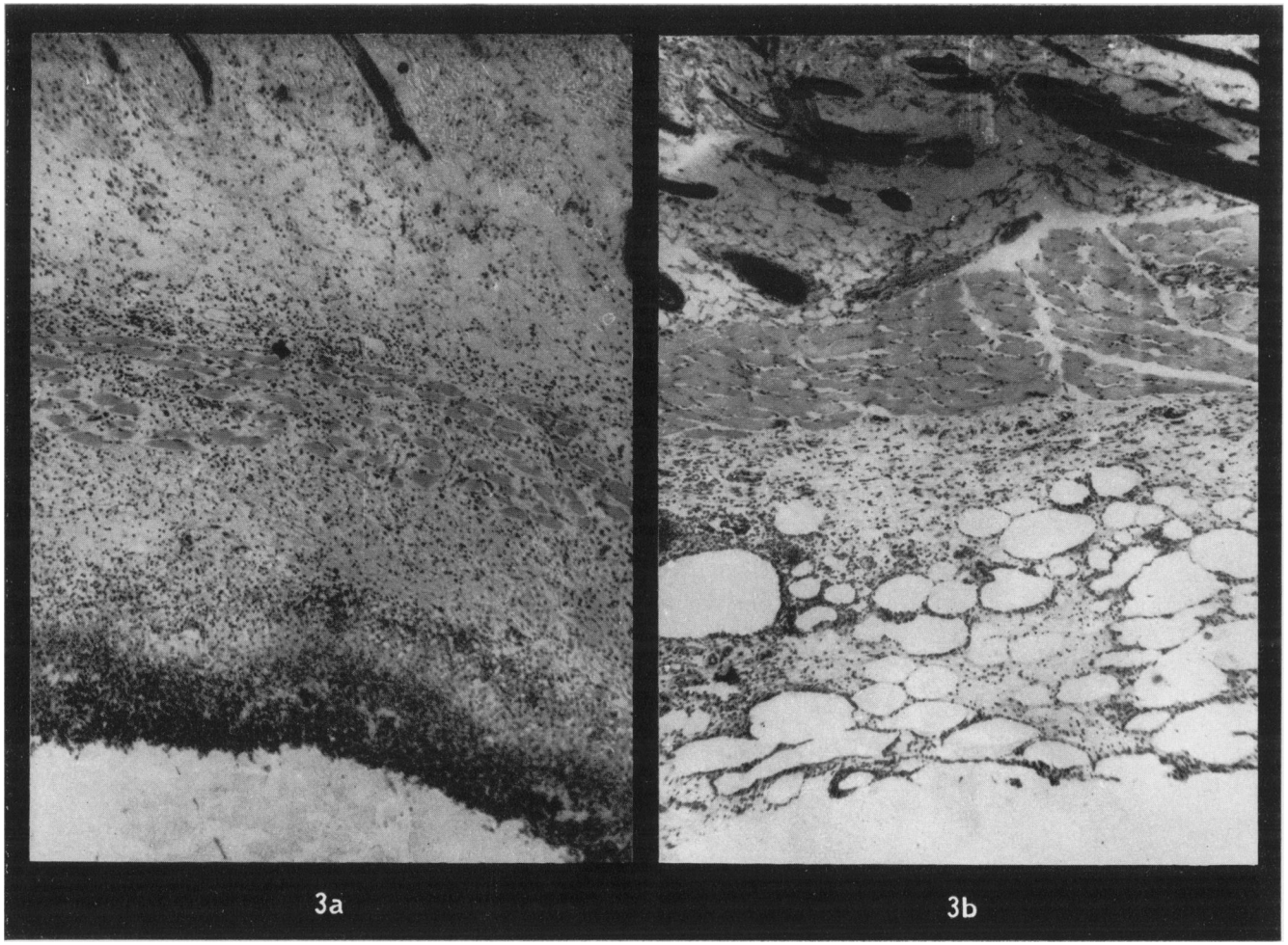
carbohydrate analyses were carried out on the seromucoid fractions from normal. capsule- and tumour-bearing rats. The limited amount of material available prevented a fuller analysis but the results shown in Table I suggest that the seromucoid fraction in the tumour + adjuvant injected animals is different from that in the tumour bearing animals, the latter being similar to the normal.

Nature of the capsule

Histological examination of the capsular material from an animal injected with Freund's adjuvant + tumour extract (Fig. 3a) indicated that it was com-

EXPLANATION OF PLATE.

FIG. 3.—Response produced by subcutaneous injection of Freund's adjuvant + tumour extract (a) and Freund's adjuvant + saline (b). H. and E. × 30.



Apsey, Burston and MacLagan.

TABLE I.—*Rat Seromucoid Carbohydrate Content*

Serum	Seromucoid protein mg./100 ml. serum	Seromucoid hexose mg./100 ml. serum	Per cent hexose
Normal (1)	102	13·8	13·5
Normal (2)	120	16·6	13·8
Freund's adjuvant + tumour extract (1)	552	84·0	15·2
Freund's adjuvant + tumour extract (2)	457·5	73·9	16·2
Walker tumour (1) bearing (10 day)	711	94·5	13·3
Walker tumour (2) bearing (10 day)	525	70·4	13·4

posed of granulation tissue with a dense cellular exudate of polymorphs and mono-nuclear cells. When Freund's adjuvant + saline (Fig. 3b) was injected the polymorph response was absent and it appeared that the adjuvant was being absorbed into the tissue, numerous fat spaces being seen at the site of injection.

In the case of the tumour + adjuvant injected animals, therefore, the capsular material appeared to be very similar to that reported by Darcy (1964) surrounding subcutaneously implanted rat kindey.

Effect of tumour injections on liver weight, liver and serum protein

The effect of tumour injection in the presence of Freund's adjuvant on liver weight, total soluble liver protein, total protein and serum glycoprotein is shown in Table II. The serum glycoprotein level was significantly increased ($P < 0{\cdot}001$) but unlike tumour growth *in vivo* there was no increase in liver weight or a decrease in total serum protein (Burston *et al.*, 1965).

TABLE II.—*Effect of Tumour Extract Injected in Freund's Adjuvant on Rat Body and Liver Weight, Liver and Serum Protein*

	No. of animals	Body wt. in g.	Liver wt. in g.	Total soluble protein g./100 g. liver wet wt.	Total serum protein g./100 ml. serum	Serum glycoprotein mg./100 ml. serum
Freund's adjuvant + saline injected	6	184	7·6±0·2	7·3±0·6	6·2±0·1	163·0±11·6
Freund's adjuvant + tumour extract injected	6	185	8·3±0·5	6·4±0·5	5·9±0·1	341·0±28·8*

*$P < 0{\cdot}001$

It was obvious from the above results that any increase in the SGP level due to delayed release of tumour extract would be obscured by the large increases associated with capsule growth. Consequently several other substances were tried as adjuvants; $Al(OH)_3$ provoked capsule formation with a corresponding increase in the SGP level, although not as great as that produced by Freund's adjuvant (Fig. 4). With $Al(OH)_3$, saline alone stimulated capsule formation while the presence of serum appeared to inhibit it, possibly being absorbed on to

the precipitate and having a protective effect. From these results it appears that the capsule formation is a response to foreign solid particles. In the case of Freund's adjuvant there is no response unless protein is also present. PVP, protamine sulphate and fibrinogen produced little capsule or rise in SGP level.

The correlation between capsule formation and increase in the SGP level is therefore a close one. This is illustrated graphically in Fig. 5. In Fig. 5a the SGP values from capsule bearing animals are compared with those from non-capsule bearing animals. The resultant graph is reminiscent of that obtained in studies of serum glycoprotein levels in human cancer (i.e. Lockey, Anderson and Maclagan, 1956) and indicates the great range of response obtained, some animals obviously being more susceptible than others. In Fig. 5b the SGP levels of those animals bearing capsules is plotted against capsule weight and it can be seen that there is a positive correlation ($r = + 0{\cdot}6$, $P < 0{\cdot}001$).

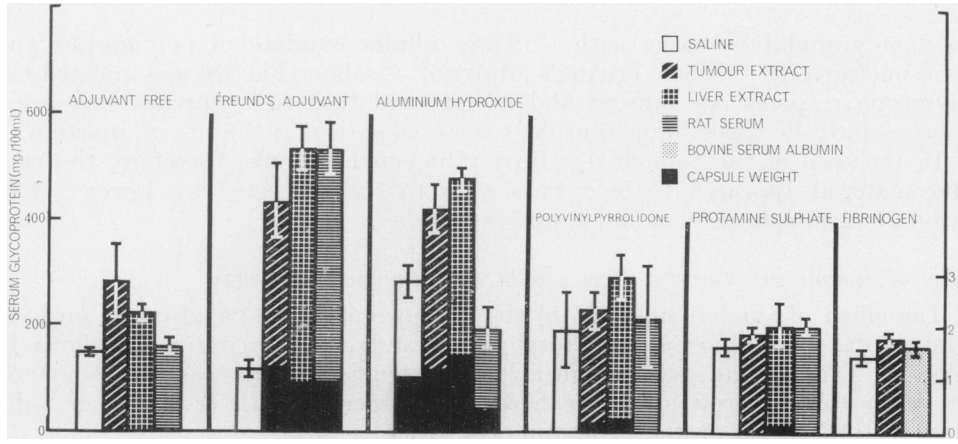


FIG. 4.—Serum glycoproteins and capsule weights 48 hr. after various subcutaneous injections (1 ml.) Each column represents mean ± S.E. of groups of 4 rats.

DISCUSSION

The effect of injected tumour extracts on the serum glycoprotein level in the rat has been studied by several workers with conflicting results. Patterson, Maxwell and McCoy (1963) found that injected Walker tumour extract would produce an increase in the serum glycoprotein level but they attributed the effect to the presence of *Salmonella typhimurium* in their extracts, sterile extracts having no effect. Similarly Darcy (1964) reported that injection of Walker tumour extract only produced a negligible increase in a specific rat serum α_1-globulin, known to increase in the tumour bearing rat. Macbeth and Bekesi (1964), on the other hand, were able to produce a significant increase in the rat seromucoid level two days after the injection of heat denatured, and therefore presumably sterile Walker tumour extract. The results reported here are therefore in accord with those of Macbeth and Bekesi in that sterile Walker tumour extracts did produce an increase in the seromucoid level. The effect produced was however small compared with that produced by the tumour *in vivo* and was non-specific, liver extract producing a similar increase.

At first sight the effect produced by capsule growth around extracts injected in adjuvant would seem to be analagous to those produced by tumour growth itself. However, the hexose estimations suggest that the composition of the seromucoid fraction which increases as a response to capsule growth is different from that which increases in response to the Walker tumour *in vivo*. This could be explained on the assumption that rat seromucoid contains at least two components of

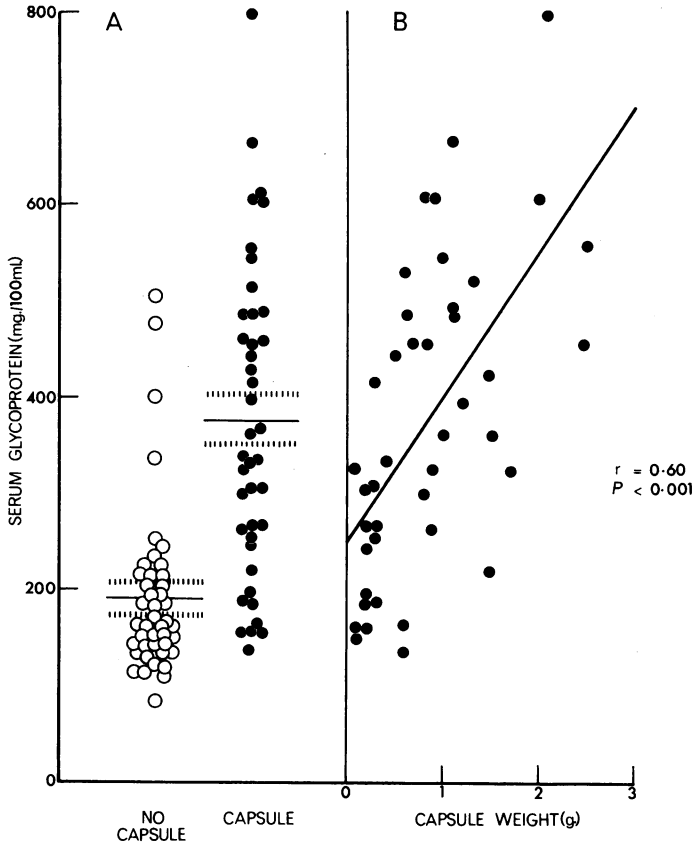


FIG. 5.—Serum glycoproteins and capsule growth. A. Serum glycoprotein levels in capsule bearing rats ●, and in non-capsule bearing rats ○. B. Correlation of capsule weight with serum glycoprotein level.

differing hexose content (Sarcione, 1963). An equal increase in both components would not alter the hexose composition whereas a greater increase in the level of one component relative to the other could change it.

Although the SGP level increases during both tumour and capsule growth, the nature of the effective stimulus is uncertain, since necrosis, rapid tissue growth and an inflammatory reaction need to be considered. There was no evidence of necrosis in the capsules, but an inflammatory reaction with round cell infiltration was present. As the latter takes place at the capsule-tissue junction it would be proportional to the surface area of the capsule and would be

correlated with growth. Darcy (1964) found an increase in a rat serum α_1-globulin with a similar encapsulation reaction around subcutaneous implants of rat kidney. Here, however, both round cell infiltration and necrosis were present. Heppleston and Keyser (1957) on the other hand reported an increase in SGP in rabbits injected intraperitoneally with silica. This produced an inflammatory response and necrosis, but in their experiments tissue growth was suppressed by cortisone. In contrast, only a small increase was found by Darcy (1964) in kidney hyperplasia where there was tissue growth without round cell invasion, and when the Walker tumour is grown ascitically where again the round cell response is comparatively small.

The most significant common factor in all systems with a positive SGP response therefore appears to be the inflammatory reaction. Whatever the actual stimulus is, it presumably acts on the liver by a humoral mechanism for which further evidence should be sought.

SUMMARY

The serum glycoproteins in the rat have been measured after the subcutaneous injection of various tumour, tissue, and protein preparations.

Increases were produced by Walker tumour and rat liver extract injected in saline.

Freund's adjuvant, $Al(OH)_3$, PVP, protamine sulphate and fibrinogen were used to produce delayed absorption of the extracts. Freund's adjuvant was the most effective, and led to the greatest seromucoid increases. Similar results were shown by tumour extract, rat liver extract, rat serum and bovine serum albumin.

A correlation was established between capsule weight and seromucoid levels, both of which may depend upon the inflammatory response.

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